Tissue integration versus bacterial colonization on dental implant materials

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CHAPTER 5

Identification of the surface properties of dental implant materials influencing tissue integration *versus* biofilm formation through backward regression analysis

Abstract

In the previous Chapters (3 and 4), we have demonstrated that the surface properties of dental implant materials can vary widely influencing biofilm formation and tissue integration in mono-culture studies as well as the outcome of co-culture experiments between contaminating and challenging bacterial strains and tissue cell. Here we aim to identify physico-chemical surface properties of dental implant materials as predictive factors with respect to the above processes through multiple linear regression analysis. The conclusion of this study is that backward linear regression analysis of data obtained from *in vitro* biofilm studies, from *in vitro* studies into adhesion, spreading and growth of tissue cells on the different implant surfaces or from *in vitro* co-culture studies on the competition between bacteria and tissue cells on dental implant materials, is unable to identify implant surface properties that control the above processes. Therewith the interplay between material properties and composition is too complex to unraffle and data can only be expressed as pertaining to a particular surface modification in combination with its chemical composition, as done in our previous Chapters 3 and 4.

Introduction

The application of dental implants in modern dentistry has provided considerably more comfort for patients in the restoration of oral function after tooth loss than traditional dental prostheses. The fate of a biomaterial implant or device can be envisaged as a race for the surface between bacteria and host tissue cells [1]. Success and failure of dental implants is directly related to the degree of integration of the implant material with the surrounding soft and hard tissues [2,3]. Physico-chemical characteristics of the implant materials such as surface roughness, hydrophobicity, and chemical composition of the implant surface, are considered as influential for tissue integration and biofilm formation [4-6]. However, no previous studies have attempted to identify a single or multiple predictive factors dominantly influencing the race for the surface between tissue integration and biofilm formation.

In the previous Chapters (3 and 4), we have demonstrated that the surface properties of dental implant materials can vary widely influencing biofilm formation and integration by human gingival fibroblasts and osteoblasts in mono-culture studies. Also, the outcome of co-culture experiments between contaminating and challenging bacterial strains and tissue cells appeared different on different implant materials. In this chapter, multivariate regression analyses are performed with the sole aim to determine the predictive factors with respect to the above processes in physico-chemical surface properties of the dental implants materials investigated in this thesis.

Materials and methods

The implant materials used and implant surface characterization methods applied have been previously described in Chapter 3. Experimental protocols and methods on supra-gingival bacterial biofilm formation, human gingival fibroblasts (HGF, ATCC-CRL-2014) culturing, and tissue cell-bacteria co-culturing have been described in Chapter 3 as well. Experimental protocols and methods on sub-gingival bacterial biofilm formation, osteoblast (U2OS, ATCC HTB-94) cells culturing and the co-culture model in which osteoblast layers were challenged with anaerobic sub-gingival bacteria have been described in Chapter 4 of this thesis.

Statistical analysis

Backward multiple linear regression analysis was performed using SPSS for Windows (Version 14.0, SPSS, Chicago IL) to identify the surface properties most predictive for biofilm formation by each single bacterial strain, HGF and U2OS cellular adhesion, spreading and growth and the outcome of the race for the surface between tissue cells and each single bacterial strain on different implant materials, both when tissue cells have to interact with a bacterially contaminated implant surface or when cells adhering

on an implant surface are challenged with bacteria. To this end biofilm volume, adhesion cell number, total surface coverage by tissue cells and its reduction due to the presence of contaminating supra-gingival bacteria or a challenge by sub-gingival bacteria were used as a dependent variable respectively, while the water contact angle, mean surface roughness, and the percentage surface composition %C, %O in oxide (metal oxide), %Ti, %Zr were entered as explanatory variables. Variables were excluded when they appeared equally weighed or correlated significantly with other variables, as determined with Pearson's correlation test, justifying the deletion of %Ti and %Zr.

In addition, backward multiple linear regression analysis was performed in which data from all four supra-gingival strains and two sub-gingival strains were combined in both mono-culture and co-culture conditions using the same explanatory variables as described above. Probability levels of 0.1 or less were considered to indicate statistical significance.

Results

Predictive factors for supra- and sub-gingival bacterial biofilm formation on different implant materials

To identify the materials properties with a significant impact on biofilm formation, the biovolume of each strain on different implant material surfaces was selected as the dependent variable (Table 1). Hydrophobicity did not significantly affect biofilm formation by *S. oralis*. Surface roughness in combination with surface compositional data, reduced biofilm formation by *S. mitis* significantly, accounting for 38% of the variation. The presence of metal oxide (either Ti or Zr), discouraged biofilm formation by *S. salivarius* and *S. aureus*, but this was in combination with surface roughness and hydrophobicity respectively. The presence of carbon content as a significant contributing factor affected anaerobic biofilm formation by *P. intermedia*, but not by *P. gingivalis*. When the averaged biofilm volume of all four supra-gingival strains was used as the dependent variable, no significant predictive factor could be identified. The presence of carbon, however, showed a significant negative effect on the combined biofilm volumes of the two sub-gingival strains, accounting however for only 29% of the variation.

Predictive factors for adhesion, spreading and growth of HGF and U2OS cells on different implant materials

To identify the materials properties with a significant impact on tissue integration, the number of adhering cells, or their surface coverage on different implant material surfaces was selected as the dependent variable (Table 2). Spread area per cell was excluded from the analysis as the total surface coverage obviously depends on the number of cells and the spread area per cell.

Table 1. Surface properties of different implant materials involved in this study with a significant influence on biofilm formation by the different supra-gingival or sub-gingival bacterial strains in mono-culture, derived from backward multiple linear regression analyses. Standardized coefficients (*β*), dependent variables and the percentages of variance explained by the independent variables (R^2) and p-values after backward multiple regression analysis. (* P < 0.1).

Strain	Variable	R^2	*β*	*P**
***Streptococcus oralis* J22**	*Water contact angle*	12%	0.344	0.192
***Streptococcus mitis* BMS**	*Surface roughness*	38%	-0.848	0.015*
	%C		-0.536	0.1
***Streptococcus salivarius* HB**	*Surface roughness*	36%	0.485	0.081*
	$\%O_{\text{in oxide}}$		-0.671	0.021*
***Staphylococcus aureus* ATCC25923**	*Water contact angle*	57%	0.514	0.000*
	$\%O_{\text{in oxide}}$		-0.571	0.014*
Combined data of all supra-gingival bacteria	*Water contact angle*	7.4%	-0.273	0.307
***Prevotella intermedia* ATCC 49046**	%C	61%	-0.784	0.0001*
***Phorphyromonas gingivalis* ATCC 33277**	-	-	-	-
Combined data of all sub-gingival bacteria	%C	29%	-0.541	0.03*

The variations observed in the number of adhering HGF cells after 48 h growth and the total surface covered could be explained for 47% and 52% with negative slopes by the combined presence of metal oxide (either Ti or Zr) and carbon (see Table 2). The variations in the number of adhering U2OS cells after 48 h growth and the total surface covered in an anaerobic condition could be explained by hydrophobicity for 41% and 38%

Table 2. Surface properties of different implant materials involved in this study with a significant influence on the number of adhering HGF and U2OS cells, or their total surface coverage, derived from backward multiple linear regression analyses. Standardized coefficients (*β*), dependent variables and the percentages of variance explained by the independent variables (R^2) and p-values after backward multiple regression analysis. (* P < 0.1).

Dependent variable	Variable	R^2	*β*	*P**
Number of adhering HGF cells per unit area	%C	47%	-0.729	0.006*
	$\%O_{\text{in oxide}}$		-0.473	0.052*
Surface coverage by HGF cells	%C	52%	-3.161	0.008*
	$\%O_{\text{in oxide}}$		-3.127	0.008*
Number of adhering U2OS cells per unit area	*Water contact angle*	41%	0.717	0.013*
	%C		-0.588	0.036*
Surface coverage by U2OS cells	*Water contact angle*	38%	0.613	0.012*

respectively. The presence of carbon showed a negative influence on the number of adhering U2OS cells but only in combination with the water contact angle.

Predictive factors for the race for the surface between HGF cells and contaminating supra-gingival bacteria on different implant materials

A similar analysis as described above was carried out taking the reduction in total surface coverage due to the presence of contaminating, supra-gingival oral bacterial strains as the dependent variable (Table 3).

Predictive factors for the growth of U2OS cells in presence of a challenge by sub-gingival, anaerobic pathogens on different implant materials

Finally, a similar analysis was carried out taking the reduction in total surface coverage due to a challenge by sub-gingival, anaerobic pathogens as the dependent variable (Table 4). When the reduction in surface coverage by U2OS cells due to a challenge with adhering sub-gingival bacteria was used as a dependent variable, the only significant

predictive factor identified is metal oxide that explained, however, only 33% of the variation when the growth of U2OS cells was challenged with *P. gingivalis*.

Table 3. Surface properties of different implant materials involved in this study with a significant influence on reduction in surface coverage by HGF cells due to the presence of adhering supra-gingival bacteria, derived from backward multiple linear regression analyses. Standardized coefficients (*β*), dependent variables and the percentages of variance explained by the independent variables (R^2) and p-values after backward multiple regression analysis. (* P < 0.1).

Reduction in cell surface coverage	Variable	R^2	*β*	*P**
HGF vs *S. oralis* J22	All variables	100%	-	-
HGF vs *S. mitis* BMS	*Surface roughness*	25%	0.502	0.139
HGF vs *S. salivarius* HB	%C	65%	-0.844	0.006*
	$\%O_{\text{in oxide}}$		-0.592	0.033*
HGF vs *S. aureus* ATCC25923	%C	22%	-0.471	0.066*
Combined data of the reduction in surface coverage	*Surface roughness*	19%	0.432	0.141

Table 3 presents the results from backward linear regression analyses, identifying implant surface properties most influential on the competition between contaminating supra-gingival pathogens and HGF cells using the reduction in surface coverage by adhering HGF as a dependent variable. All independent variables together including surface roughness, hydrophobicity, carbon and oxygen in metal oxide affected the surface combat between *S. oralis* and HGF cells, accounting for 100% of the variation in the reduction in surface coverage of HGF cells. Carbon in combination with oxygen in oxide is a significant predictive factor for the reduction in surface coverage of HGF cells at the presence of *S. salivarius*, explaining for 65% of the variation.

Discussion

TiZr alloy and ZrO_2 are increasingly being considered as alternatives for Ti dental implants, along with different surface modifications of Ti implant materials to stimulate tissue integration and prevent bacterial colonization of the surfaces. In this study, we have analyzed the effects of physico-chemical properties of implant material surfaces on

biofilm formation, tissue integration and competition between tissue cells and oral pathogens. Although several *in vitro* and *in vivo* studies pointed to an influence of surface roughness and/or hydrophobicity on bacterial colonization [7-9], our study did not identify a single implant material surface property that showed a ubiquitous influence on biofilm formation across all four supra-gingival strains, although roughness and hydrophobicity in combination with the presence of metal oxide appeared as being highly influential on average biofilm formation by all four supra-gingival strains. Biofilm formation by anaerobic *P. intermedia* or across all two sub-gingival bacteria, in contrast, appeared to be influenced only by carbon content.

Regression analysis was never able to explain more than 52% of the variation observed in HGF cell adhesion and growth or more than 38% of the variation obsverved in U2OS cell growth between the present collection of implant materials, with the presence of metal oxide in combination with carbon or water contact angle as the most predictive factors respectively. The presence of carbon content, intriguingly, affected in a similar way the results of U2OS cells adhesion, although it would be anticipated that a cleaner surface (less carbon) would yield a higher exposure of the metal. Overall, the lack of effect of implant material type on the surface coverage by HGF cells found in this *in vitro* study coincides with results from clinical studies, showing no differences in soft tissue health of peri-implant mucosa adjacent to ZrO_2 and Ti abutment surfaces [10].

"Mono-cultures" studies in which bacteria and cells were not in competition with each other, as they are in a clinical situation, have not unequivocally yielded a single implant surface property that determined either biofilm formation or soft and hard tissue cells adhesion, spreading and growth (data from mono-culture experiments in Chapters 3 and 4). However, also in this chapter in which backward multiple linear regression analyses were performed on data from both mono-culture and co-culture models, we could not reveal predominant implant surface properties that could explain the outcome of the competition between bacteria and tissue cells. By comparison with the electron micrographs and properties of the implant surfaces presented in Chapter 3, it can be seen that roughness in combination with the morphology of the implants surfaces may control their interaction with bacteria and tissue cells. Data clearly indicate a chance for HGF cells to win the race for the surface on smooth, unstructured surfaces. Neither changing the implants surface composition by alloying Ti and Zr, or the use of ZrO_2, nor creating a more hydrophilic implant surface mattered in this competition. In Chapter 4 we demonstrated that U2OS-osteoblasts on average withstand a challenge by sub-gingival strains better on TiZr alloys and ZrO_2 than on Ti variants regardless of roughness indicating an overriding effect of material chemistry [11]. However, a high correlation between Ti% and Zr% as explanatory variables were revealed by Pearson's correlation test, resulting in the exclusion of Ti% and Zr% from the regression analysis, that conseqnently failed to

determine the predominant factor affect the outcome of the final competition between sub-gingival bacteria and U2OS cells.

Summarizing, the conclusion of this study is that backward linear regression analysis of data obtained from *in vitro* biofilm studies, from *in vitro* studies into adhesion, spreading and growth of tissue cells on the different implant surfaces or from *in vitro* co-culture studies on the competition between bacteria and tissue cells on dental implant materials, is unable to identify implant surface properties that control the above processes. Based on inspection of the data and without performing any formal analyses, it is considered unlikely that other types of e.g. non-linear analyses will yield a single, or multiple implant surface properties that control these processes. This frustrating conclusion coincides with the confusion lingering in the literature on whether or not hydrophobicity or roughness of a dental implant material reduces the risk of developing a peri-implant infection in patients. Therewith in the meantime, the only conclusion is that the interplay between material properties and composition is too complex to unraffle and data can only be expressed as pertaining to a particular surface modification in combination with its chemical composition, as done in our previous Chapters 3 and 4.

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